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(72) Inventor KENNETH JOHN PARKER

(54) SWEET SUBSTANCE

(71) We, TATE & LYLE LIMITED, a British company of 21 Mincing Lane, London E.C.3, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

This invention relates to a sweet substance which is useful as a sweetener for edible materials.

Although sucrose is still the most widely used sweetening agent, synthetic sweeteners, such as sodium saccharin and sodium cyclamate, have become increasingly important in recent years. These synthetic sweeteners, which are several hundred times as sweet as sucrose, are especially useful when it is desired to combine a high degree of sweetness with a low calorie content, for example in dietetic preparations and in the manufacture of "soft drinks". However, some concern has been expressed about the toxicity of synthetic sweeteners, and the use of sodium cyclamate has been restricted or banned in some countries.

Accordingly, there is a need for an alternative sweetening agent to sucrose, possessing the advantage of synthetic sweeteners such as sodium saccharin and sodium cyclamate, but without their concomitant disadvantages. The present invention provides such a sweetening agent.

The sweet substance of the invention can be produced from the fruit of the plant *Dioscoreophyllum cumminsii* Diels (syn. *D. lobatum*) of the family *Menispermaceae*. This vine-like plant, which is native to the forests of tropical West Africa, bears grape-like clusters of red berries about ½ inch in diameter; and these fruit have become known as "Serendipity Berries". The berries have a tough outer skin, enclosing a white mucilaginous material surrounding the seed.

Although it is well known that Serendipity Berries are intensely sweet, and the sweet principle can be extracted from the pulp of the berries with water, it is not possible to isolate a commercially useful sweetening agent from such an extract, since the sweet principle is unstable. For example, the sweet principle in the extract is destroyed after a time by naturally occurring enzymes. Also, the sweet principle is labile at elevated temperatures, being immediately destroyed on boiling, so that the extract or a foodstuff containing it cannot be pasteurized without losing its sweetness.

It has now surprisingly been found possible, by a series of enzymatic degradation and separation steps, to produce from Serendipity Berries an intensely sweet substance of relatively low molecular weight, which is thermally stable, and which is useful as a sweetening agent for edible materials. Also surprisingly, it has been found that this sweet substance is of proteinaceous character.

Accordingly, the invention provides a sweet substance, derivable by enzymatic degradation from the mucilaginous portion of the berries of *Dioscoreophyllum cumminsii*, having a molecular weight not greater than 10,000 and comprising a polypeptide which includes units from the following amino acids: alanine, α-aminobutyric acid, γ-aminobutyric acid, arginine, aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine.

Depending upon the degree of enzymatic degradation employed, the sweet substance of the invention may have a molecular weight of about 8,500 or, preferably, about 6,000 (the molecular weights are estimated by gel chromatography). When pure it is a colourless solid, which is freely soluble in water, both cold and hot, but insoluble in common organic solvents (e.g. acetone, ethanol, benzene, chloroform and petroleum ether); and it is precipitated from aqueous solution by ethanol and by ammonium sulphate. It is stable even at elevated temperatures: for example, an aqueous solution of it can be boiled for one

hour without destroying the sweetness. Its infra-red and ultra-violet spectra show absorptions characteristic of a polypeptide.

The process for producing the sweet substance of the invention from Serendipity Berries makes use of at least two enzymatic steps. In the first of these, the mucilaginous pulp of the berries is digested with a pectinase, so as to allow the extraction of the sweet principle; and, in the subsequent enzymatic step or steps, the sweet principle is subjected to the action of a proteolytic enzyme, such as papain or bromelain. These enzymatic steps are carried out under the conditions conventionally employed for the enzymes concerned. After each enzymatic step, the sweet principle can be purified by the techniques conventionally used for the purification of proteins, for example selective precipitation or chromatography.

The initial extraction and treatment with pectinase can be performed before or after the sweet pulp of the berries has been separated from the skins and seeds. For example, the skins can be removed from the berries, and the remaining mucilaginous pulp and seeds incubated with pectinase; or the pulp can be separated from the skins and seeds, diluted with water and digested with pectinase; or the whole berries can be homogenized in water, and the mixture digested with pectinase. Whichever method is used, care should be taken not to break up the bitter seeds. The product of the pectinase treatment is filtered, to remove solid materials such as remaining seeds and cell debris. The filtrate containing the sweet principle can be concentrated under reduced pressure, and it can be lyophilized; but, at this stage, the sweet principle is not stable at elevated temperatures, and so the filtrate cannot be boiled without destroying the sweetness.

The sweet extract thus obtained is then fractionated, for example by precipitation with ammonium sulphate or by chromatography on Sephadex ("SEPHADEX" is a Trade Mark for a chromatographic medium derived from dextran). The fraction containing the sweet principle, which can be detected by taste, is then subjected to the proteolytic enzyme treatment, in one or more stages. A controlled proteolysis is employed, since it is not intended to achieve complete degradation of the sweet principle. It is generally found that the proteolysis will proceed to a natural end point for the enzyme. If it is then desired to continue the degradation, the sweet principle is separated and subjected to a further stage of proteolysis, for which it may be advantageous to employ a different enzyme. In this way, the proteolytic treatment can give first a sweet substance with a molecular weight of about 8,500 and then a sweet substance with a molecular weight of approximately 6,000, although this latter can also be obtained by a single proteolytic stage. After each stage of the enzyme treatment, the sweet substance can be purified as before, for example by using ammonium sulphate precipitation or chromatography on Sephadex ("SEPHADEX" is a Trade Mark); and the product can be further purified by adsorption on an ion-exchange cellulose, such as carboxymethylcellulose. A solid product can be obtained from solution by evaporation or lyophilization.

The sweet substance of the invention has many advantages by comparison with the sweeteners previously used as alternatives to sucrose. Depending upon the method of evaluation used, it is estimated to be up to 3,000 times as sweet as sucrose, and thus much sweeter than saccharin. It does not have the unpleasant, bitter after-taste characteristic of synthetic sweeteners such as saccharin and cyclamate and, being derived from a natural source, it is generally more acceptable than such synthetic sweeteners. It also does not have undesirable side-flavours, unlike some other sweetening agents which have a phenolic taste, and also unlike the liquorice flavour of a simple aqueous extract from Serendipity Berries themselves. Also unlike other sweetening agents which have been investigated, its sweet taste is perceptible immediately, but it does not leave a persistent sweet taste in the mouth, which would taint anything subsequently eaten.

Accordingly, the substance of the invention may be used to sweeten edible materials in general, including all kinds of foodstuffs, beverages and pharmaceutical preparations. For ease of addition, and in order to achieve homogeneous distribution at the desired dilution in the edible material, the sweet substance of the invention may be formulated in the conventional manner with solid or liquid non-toxic carriers and diluents, to provide sweetening compositions of the desired concentration. For example, solid compositions may take the form of tablets or powders, using edible solid carriers such as lactose, starch, or nutritive proteins (e.g. soy protein); or the sweet substance of the invention can be mixed with sucrose, to provide a "fortified" sugar. Liquid compositions may take the form of aqueous solutions, or of suspensions in other non-toxic liquids such as ethanol, glycerol and edible oils.

The invention is illustrated by the following Examples. The percentage concentrations of all the solutions mentioned in the Examples are calculated on a weight/volume basis.

Example 1.

Preparation of sweet substance.

Serendipity Berries were washed and their skins were removed, leaving the mucilaginous pulp and the seeds. The mucilage was treated with pectinase, then filtered through an Oxoid membrane ("OXOID" is a Trade Mark) and

concentrated. The concentrate was applied to a column of Sephadex G—50 ("SEPHADEX" is a Trade Mark) (Dextran blue: 120 ml.; potassium chromate: 363 ml.) which had been equilibrated with a 0.01 M sodium chloride solution. The column was eluted with the same solution, at the rate of 0.5 ml. per minute. The sweet fraction was eluted at 140—180 ml, corresponding to a molecular weight of about 10,000.

The sweet fraction was evaporated to dryness under reduced pressure, and the 150 mg. of residue taken up in 10 ml. of acetate buffer (pH 4.6). The solution was treated with 1.5 ml. of a 0.02% by weight solution of bromelain activated with cysteine hydrochloride, and was incubated for 16 hours at 25° C. The digest was fractionated on a Sephadex G—50 column ("SEPHADEX" is a Trade Mark) and found to elute at 180—250 ml. The bromelain treatment was repeated twice more, using larger amounts of bromelain, followed in each case by chromatography on Sephadex G—50 ("SEPHADEX" is a Trade Mark). The sweet residue obtained after each enzyme treatment amounted to 100 mg., 40 mg. and 17 mg., respectively. The elution position of the sweet fraction after each treatment remained constant, corresponding to a molecular weight of about 8,500, showing that a sweet polypeptide of this molecular weight represents a natural end point for the enzyme, further enzymatic action merely resulting in complete hydrolysis.

This sweet fraction was incubated with cysteine hydrochloride activated papain for 24 hours at 27° C. The digest was fractionated on a Sephadex column; the papain treatment was repeated; and the digest was again fractionated on Sephadex ("SEPHADEX" is a Trade Mark). The product had a molecular weight of approximately 6,000.

The polypeptide in this product was analysed for its amino acid content. The product was treated with 6N hydrochloric acid at 100° C for 24 hours. The hydrochloric acid was removed; and the amino acid content of the concentrated hydrolyzate was analysed on a Technicon automatic analyser resin column. The following results were obtained:

Amino Acid	Mol. wt.	Micromoles in Original Solution	% Amino Acid on Total Sample
Aspartic Acid	133.1	0.085	9.4
Threonine	119.1	0.059	6.5
Serine	105.1	0.031	3.4
Glutamic acid	147.1	0.094	10.4
Proline	115.1	0.073	8.0
Glycine	75.1	0.095	10.5
Alanine	89.1	0.040	4.4
α-Aminobutyric acid	103.1	0.009	1.0
Valine	117.1	0.018	2.0
γ-Aminobutyric acid	103.1	0.027	3.0
Cystine	240.3	0.008	0.9
Iso-Leucine	131.2	0.024	2.6
Leucine	131.2	0.062	6.8
Tyrosine	181.2	0.032	3.5
Phenylalanine	165.2	0.051	5.6
Lysine HCl	182.7	0.114	12.6
Arginine	210.7	0.063	6.9

Example 2.

Preparation of sweet substance.

225 g. of Serendipity Berries were washed, and their skins were removed and discarded. The remaining 92 g. of mucilaginous pulp and seeds were incubated overnight at 27° C with 0.6 g. of pectinase. The seeds, weighing 39 g. were then filtered out, leaving 53 g. of sweet mucilage digest. This was freeze-dried, to give 7 g. of a cream-coloured powder.

The freeze-dried product was dissolved in 100 ml. of water, and was fractionated by adding solid ammonium sulphate to the solution with vigorous stirring, in the following stages:

1. 16 g. ammonium sulphate (20% saturation) — the precipitate obtained was centrifuged off and discarded;
2. 4 g. ammonium sulphate (25% saturation) — very little precipitate was formed, which was also discarded;
3. 4 g. ammonium sulphate (30% saturation) — the precipitate obtained was again centrifuged down and discarded;
4. 16 g. ammonium sulphate (50% saturation) — a large amount (3.75 g.) of brown, rubbery, sweet-tasting precipitate was obtained, and this was collected.

This last precipitate was washed very quickly with cold water, and dissolved in 50 ml. of water with stirring. The pH of the solution was adjusted to 6, 0.10 g. of bromelain and 0.02 g. of cysteine hydrochloride were added, and the mixture was incubated overnight at 27° C. The digest was again fractionated with ammonium sulphate, as above, yielding 0.90 g. of a sweet precipitate. Chromatography on Sephadex G—50 ("SEPHADEX" is a Trade Mark) showed this to have an average molecular weight of about 6,000.

The 0.90 g. of sweet precipitate was dissolved in 8 ml. of water and fractionated on a column of Sephadex G—25 (volume 130 ml.) ("SEPHADEX" is a Trade Mark) packed in distilled water, in order to separate the sweetener from sodium chloride and ammonium sulphate. The salt-free sweet substance thus obtained was further concentrated and purified by adsorption onto an ion-exchange cellulose. For this purpose, a column was packed with carboxymethylcellulose CM—32 (volume 25 ml.) in the sodium form, and equilibrated with molar urea solution. The sweet substance was applied to this column and eluted with sodium chloride solution in M urea of increasing strength. Three fractions exhibiting ultra-violet absorption at 275 nm were obtained, but only the fraction eluted with 0.1 M sodium chloride was sweet. This fraction was freed from urea and salt by chromatography, as above, and the product was freeze-dried, to give 0.025 g. of a chromatographically homogeneous substance with a very intense sweet taste.

A sample of the freeze-dried purified product thus obtained was incorporated into a potassium bromide disc, and its infra-red absorption spectrum was recorded. The spectrum showed absorptions typical of a polypeptide, including the following:

Unbonded NH_2 at 3420 cm^{-1}
Hydrogen bonded NH at 3300 cm^{-1}
NH stretching at 3070 cm^{-1}
Amide (I) band at 1660 cm^{-1}
Amide (II) band at 1535 cm^{-1}.

The ultra-violet spectrum of the substance was also recorded, at a concentration of 0.75 mg./ml. in water. This showed an absorption at 275 nm which is characteristic of polypeptides containing aromatic amino acids. This absorption peak had a shoulder at 283 nm.

Example 3.

Organoleptic evaluation of sweet substance.

These evaluations were all carried out on the pure, freeze-dried sweet substance having a molecular weight of approximately 6,000 (as estimated by gel chromatography), obtained by the procedure of Example 2.

The sweetness of the substance was first estimated in solution in distilled water, using two methods: (a) by comparison with sucrose, and (b) by determination of its threshold sweetness level.

(a) Comparison method

As series of test solutions (I—IV) was prepared, containing sucrose dissolved in water at various concentrations. A solution (x) of the sweetener of the invention in distilled water was also prepared, having a sweetness bracketed by the range of sucrose solutions. A panel of 17 tasters was then asked to match the sweetness of solution (x) to that of the sucrose solutions (I—IV).

The test solutions used had the following concentrations:

I	5% sucrose
II	10% sucrose
III	16% sucrose
IV	20% sucrose
x	0.0062% sweetener of invention.

The following results were obtained:

Proportion of tasters	Sweetness evaluation
40%	$II > x \geqslant I$
40%	$IV > x \geqslant III$
20%	$III > x \geqslant II$

None of the tasters placed the sweetness of x below that of solution I or above that of solution IV. Consequently, on the basis of these evaluations, the sweet substance of the invention at a concentration of 0.0062% had a sweetness equivalent to sucrose in a concentration range of 5%—20%. On a weight

basis, this means that the sweet substance of the invention had a sweetness 800—3,200 times that of sucrose.

(*b*) *Threshold method*

Samples of solutions of the sweet substance of the invention in distilled water (B—D), and a control sample consisting only of distilled water (A), were evaluated by a panel of 15 tasters, who were asked to state whether each sample was sweet. The following results were obtained:

Sample	Concentration	No. of times recognised as sweet
A	0.0 (water control)	0
B	2.48 ppm	3
C	6.20 ppm	12
D	12.40 ppm	13

From these results, it appears that the sweet substance of the invention has a threshold of sweetness:

for 20% of tasters—2.48 ppm.
for 80% of tasters—6.20 ppm.
Average threshold level—4 ppm.

The threshold of sweetness for sucrose is about 7,000 ppm; and, on this basis, the sweet substance of the invention has a sweetness approximately 1,750 times that of sucrose.

(*c*) *Qualitative organoleptic evaluation*

The taste of the sweet substance of the invention was also assessed qualitatively, (i) in distilled water solution, (ii) in admixture with a soybean material, and (iii) in grapefruit juice.

(i) A panel of 17 tasters described the taste of the sweetener of the invention in distilled water solution as follows: pleasant; taste lingers fractionally longer than sucrose; saccharin-like after-taste but not bitter; distinguishable from sucrose but not unpleasant; artificially sweet; thin, not full-bodied; distinct after-taste, but not unpleasant; ordinary.

(ii) A water-soluble, bland soy protein isolate was dissolved in water, and an aqueous solution of the sweet substance of the invention, as used in the previous tests, was added to it. The sweetened solution was freeze-dried. When the freeze-dried product was redissolved in water, it was found to be as sweet as the original aqueous solution of the sweet substance, without any interference from the soy protein.

(iii) A few drops of the solution of the sweet substance of the invention, as used in the previous tests, was added to an unsweetened grapefruit juice. Organoleptic evaluation showed that the juice had been satisfactorily sweetened, and the sweetness was not destroyed by the grapefruit sourness.

WHAT WE CLAIM IS:—

1. A sweet substance, derivable by enzymatic degradation from the mucilaginous portion of the berries of *Dioscoreophyllum cumminsii*, having a molecular weight not greater than 10,000, and comprising a polypeptide which includes units from the following amino acids: alanine, α-aminobutyric acid, γ-aminobutyric acid, arginine, aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine.

2. A substance according to claim 1, having a molecular weight of about 8,500.

3. A substance according to claim 1, having a molecular weight of about 6,000.

4. A process for producing a sweet substance according to claim 1, which comprises digesting the mucilaginous portion of the berries of *Dioscoreophyllum cumminsii* with pectinase, separating a sweet proteinaceous fraction from the pectinase digest, and treating this sweet fraction with a proteolytic enzyme.

5. A process according to claim 4, in which the treatment with a proteolytic enzyme is carried out in at least two stages, the second and any subsequent stage being carried out on a sweet fraction separated from the product of the previous stage.

6. A process according to claim 4 or claim 5, in which the proteolytic enzyme is papain or bromelain.

7. A process according to any of claims 4 to 6, in which the separation of the sweet fraction is effected by means of selective precipitation or chromatography.

8. A process according to any of claims 4 to 7, in which the treatment with the proteolytic enzyme is continued until a sweet substance having a molecular weight of approximately 8,500 is obtained.

9. A process according to any of claims 4 to 7, in which the treatment with the proteolytic enzyme is continued until a sweet substance having a molecular weight of approximately 6,000 is obtained.

10. A process according to claim 4, substantially as herein described in Example 1 or Example 2.

11. A sweet substance according to claim 1 when produced by a process according to any of claims 4 to 10.

12. A sweet composition comprising an edible material and a sweet substance according to any of claims 1 to 3 or 11.

MARKS & CLERK,
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57 & 58 Lincoln's Inn Fields,
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